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TECHNOLOGY ASSESSMENT AND STRATEGY FOR DEVELOPMENT OF A RAPID FIELD WATER MICROBIOLOGY TEST KIT

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A literature and market search of existing technology for the detection, identification, and quantification of microorganisms in water was conducted. Based upon the availability of technologies and their configurations, an assessment of the appropriate strategies to pursue for the near and long term development plans in development of the Rapid Field Bacteriology Test Kit was performed. Near term technologies to improve the Army's capability to detect microorganisms would appear to be essentially improvements in versatility and measurement of coliform indicator organisms. New chromogenic and fluorogenic indicator substances associated with new substrates appear to be best suited for test kit development either for quantitative membrane filter tests or presence/absence and multiple fermentation tests. Test times, incubator requirements, and operator involvement appear to be similar to older technologies.

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Long term development would appear to favor such technologies as genetic probes with amplification of the hydridized nucleic acid materials of positive samples, and some immunological based systems such as enzyme linked, immuno-sorbent assays. In both cases, the major problems would appear to be sample preparation and development of signal strengths from the reactions which would allow the user to see results in 1 hour. Packaging and electrical requirements may also be problematic.

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INTRODUCTION

Throughout man's history the outcome of wars, great and small, has often hinged on the health of the soldier and the associated incidence of diseases of the gastrointestinal tract which often are caused by pathogenic waterborne microorganisms. Even today the food and water that we consume may be highly contaminated with infectious bacteria, viruses, fungi, protozoa, and helminths, which when consumed may lead to severe, debilitating illness and even death. The dependence of soldiers on the battlefield for safe water, therefore, is of prime interest to the preventive medicine community; and this community has a major mission in the prevention of disease from contaminated water (and foodstuffs). The main tools that preventive medicine field personnel have to prevent waterborne illnesses are methods to measure the disinfectant residuals in water to make sure water is satisfactorily disinfected (5-10 mg/L of free available chlorine for 30 minutes), or the determination of indicator bacteria (coliform organisms using membrane filter or most probable number techniques for which a maximum of 1 coliform organism/100 ml of water is allowed to ensure that other pathogenic microorganisms are not present).

Water disinfection is typically utilized as the second barrier to pathogenic microorganisms after water filtration by Erdlator or reverse osmosis water purification units. While disinfection is a very important barrier and very effective for almost all microorganisms at the point of water treatment, it is often difficult to ensure that the disinfectant will remain in the water for prolonged periods, especially after storage and subsequent multiple distribution procedures in the field. Often disinfectant residual is lost and is difficult to reapply in storage and distribution equipment, thus opening the door to microbiological recontamination of water before it reaches the individual soldier.

The coliform organisms are a well recognized surrogate or indicator of microbiological contamination of water; but they have some significant shortcomings such as: (1) they do not adequately indicate the effectiveness of water treatment nor the presence of enteric viruses and protozoan cysts; (2) they may not accurately reflect fecal contamination especially in tropical climates since the coliforms can grow in pristine waters and on vegetation in such climatic regions; (3) they require an 18 to 24-hour incubation period before it can be ascertained whether or not contamination exists and verification may require another 24 to 48 hours. Military personnel often have to consume the water within periods much less than 24 hours after treatment; (4) Other mammals shed coliform organisms so their presence alone may not be a good indication of the presence of human pathogens.

Because of shortcomings in the determination of chlorine at various water treatment, storage, and distribution points and the limited effectiveness of current microbial indicator organisms, the Academy of Health Sciences prepared an Operational and Organizational (O&O) Plan recognizing the need to develop improved methods to detect microbiological and chemical parameters in water. The title of this O&O Plan is "A Family of Medical Water Quality Monitoring Equipment (FMWQME)." This report presents the results of an in-house literature and market survey of microbiological testing procedures available

on the market or proposed for development in the future to meet the O&O Plan requirements for one component of this family of equipment, namely the "Rapid Bacteriological Test Kit (RABTEK)." This report also reflects a basic research strategy for the development of RABTEK technology.

The specific needs for the FMWQME as stated in the O&O Plan are as follows:

a. A family of equipment to monitor health related water quality parameters is needed to provide for a simplified, rapid determination of the potability of field water. These parameters will include, but are not limited to, organic substances, inorganic substances, and pathogens. Limited capabilities exist in this area, but are outdated wet chemistry procedures that use chemicals with a short shelf life. In addition, the sets are large and bulky. The current bacteriological test kit is too complicated and slow to support the fast moving Airland Battlefield. A new kit that is simpler and more rapid than the current water test kit will decrease the time it takes to get the results back to the commander. Waterborne disease threats pose significant operational concerns to commanders. This deficiency describes the need for a capability to rapidly test existing water sources for potability. This family is not intended to replace the M 272E2 Water Test Kit, Chemical Agent, which needs to be included on preventive medicine unit Table of Organization and Equipments (TOEs). A test kit to measure radiological contamination in water also is needed but a proponent school has not yet been identified to develop this kit.

b. The basis for this need was identified as Issue #2 in the 1988 Mission Area Development Plan Update of Combat Service Support Mission Area Analysis, Part 8, Medical, as "Inadequate capability to assess, prevent, and treat endemic diseases and other environmental health threats."

This report is divided into four separate sections dealing with: a discussion of microorganisms of concern in water; a review of classical and new detection systems; novel new approaches for microbial detection; and conclusions and recommendations for new preventive medicine capabilities for the detection of pathogens (and their indicators) in water supplies.

MICROORGANISMS OF CONCERN IN DRINKING WATER

The normal microbiological flora of the human gastrointestinal tract may be regarded as either "the more intimate portion of the environment or as an organ of the body" (Draser and Barrow 1985). If one considers the flora as an organ, it is highly diverse in both a biochemical and pathological sense. In addition to the normal bacterial flora which are required for an individual to remain healthy (Table 1), several pathogenic microorganisms may invade the gastrointestinal tract, replicate, be excreted, and enter into an explosive epidemiological cycle whose magnitude is not buffered by the less than stringent sanitary and hygienic practices of soldiers under field conditions. These etiological agents include bacteria, viruses, parasites, fungi, and helminths whose properties make them suitable to be transmitted by the oral or fecal-oral route. The source of enteric infection can be the consumption of contaminated water, exposure to aerosols, improperly slaughtered or prepared

meats, raw fruits or vegetables cultivated on contaminated land or washed with contaminated water. The origin of infectio may be man or other mammalians including livestock and wildlife and also birds. This chapter will concentrate on the etiological agents transmitted by the oral or fecal-oral route, as well as their indicators, which may be found in water destined for human consumption.

Pathogenic bacteria which invade the human gastrointestinal tract must exhibit properties which allow survival in this extreme environment. They must be able to withstand the detergent action of bile salts, utilize available nutrients, and survive under anaerobic conditions either as a strict anaerobe or as a facultative organism. These bacteria may or may not form spores and, therefore, have different capabilities to survive outside of the gastrointestinal tract. Genera which may cause gastroenteritis are presented in Table 2. These bacteria cause disease mainly by the production of endo or exotoxins. The infective dose of pathogenic bacteria is notably high, as shown in Table 3.

Enteric viruses come from several different groups and cause a broad spectrum of disease syndromes (Table 4). The most extensively studied enteric viruses are the three types of poliovirus. This virus has come to represent a prototypical enteric virus. Its structure is a 20 nm diameter icosahedron containing a single-stranded ribonucleic acid (RNA) genome. The icosahedron consists of protein and is devoid of lipid, which tends to make this type of virus suited for survival within the environment for extended periods of time. Unlike bacteria, viruses cannot replicate outside of their host. These agents cause disease mainly by cell destruction as a consequence of their replication within a host cell. A particularly unnerving aspect of viruses in the environment is that their infective dose is extremely low, on the order of one to one hundred infectious virus particles.

The parasites of humans are classified in the kingdom PROTOZOA with three phyla: (1) ARCOMASTIGOPHORA, which contains the flagellates and amebas, (2) APICOMPLEXA, which contains the sporozoans, and (3) CILIOPHORA, which contains the ciliates (Heyneman 1987). Subphyla of SARACOMASTIGOPHORA, which contain human genitourinary and intestinal parasites include: (1) MASTIGOPHORA, the flagellates, including Giardia, Trichomonas, Petortamonas, Dientamoeba, Enteromonas, and Chilomastix (Heyneman 1987), (2) SARCODINA, which are typically ameboid, including Entamoeba, Endolimax, Iodamoeba, Naeleria, and Acanthamoeba (Biddeik et al. 1984; Heyneman 1987), (3) SPOROZOASIDA, whose life cycle involves alternating sexual and asexual reproductive phases usually involving two hosts, including the subclass COCCIDIA which contains the important human intestinal parasite Cryptosporidium (Heyneman 1987; Fayer and Unger 1986; Levine 1984), and (4) CILIOPHORA, complex protozoa bearing cilia and two kinds of nuclei, includes Balantidium coli, the giant intestinal parasite of humans and pigs (Heyneman 1987).

Human parasitic worms, or helminths, are classified as belonging to two phyla, PLATYHELMINTHES and NEMATHELMINTHES. Medically important members of the phylum PLATYHELMENTHES, the flatworms, belong to the classes: (1) CESTODA (tapeworms) whose important members include the genera Diphyllobothrium, Spirometra, Taenia, Echinococcus, Hymenolepis, and Diphylidium, and (2)

TREMATODA (flukes) whose important members include the genera Schistosoma, Paragonimus, Clonorchis, Opisthorchis, Heterophyes, Metagonimus, Fasciolopsis, and Fasciola (Heyneman 1987). According to Science, Schistosoma is public enemy number two on the World Health Organization's list of top ten scourges (Pool 1989). Medically important members of the phylum NEMATHELMINTHES, the roundworms, transmitted by the fecal-oral route are numerous to the extent that they are presented in Table 5, along with other helminths.

Human intestinal fungi such as Candida, as well as other yeasts, are normal members of the intestinal flora, but can become invasive in an immunocompromised host (Jawetz 1987). Recently, Wangiella dermatitidis was isolated from edible mushrooms (Kazanas 1986). The possibility of such lethal fungal infections being transmitted by the oral route was demonstrated in this report. Several fungi, including Histoplasma, find their niche in the soil and on rotting wood and may become human pathogens of the upper respiratory tract (Lavie and Stotzky 1985; Lavie and Stotzky 1986a). Many of these fungi are dermal in nature and can invade wounds or other breaks in the skin (Jawetz et al. 1987). Therefore, these agents represent a potential public-health threat if they are present in drinking, shower, or surgical water.

Micro and macroinvertebrates of the taxonomic groups CRUSTACEA, NEMATODA, PLATYHELMINTHES, and INSECTA are found in potable water distribution systems (Levy et al. 1984). These organisms can cause problems with taste, odor, water discoloration, filter clogging, and high turbidity. MacDenthun and Keup (1970) indicated that these organisms pose no public health threat, whereas others (Haney 1978; Levy et al. 1984) report that that these animals may ingest pathogenic bacteria prior to being ingested by humans and transfer pathogenic organisms in this manner. Gerardi and Grimm (1982) and King et al. (1988) reported that these organisms could obscure pathogenic bacteria from detection, protect these microorganisms from disinfection, and allow them to persist in distribution waters.

Due to the fact that several of the more highly pathogenic bacteria exist in very low levels in fecally-polluted water and that it would be economically disastrous to assay waters for all possible pathogens, the concept of indicator organisms of fecal pollution of water has been introduced. The coliform group of bacteria includes members of the genera Enterobacter, Escherichia, Citrobacter, and Klebsiella (Covert et al. 1989); all of which are normal inhabitants of the human gastrointestinal tract. More practically, the coliform group comprises all aerobic and facultative anaerobic, gram-negative, nonspore-forming, rod-shaped bacteria that ferment lactose with gas production within 48 hours at 35°C (American Public Health Association 1985). A more rigorous indicator is the fecal coliform test which uses a selective and differential medium at 44°C to determine the presence of bacteria from the intestines of warm-blooded animals which can utilize lactose and produce gas (American Public Health Association 1985). It is interesting to note that Escherichia coli, which has traditionally been the prototypical enteric bacterium, represents less than 1 percent of the bacterial mass in human fecal material (Drasar 1985). Fecal streptococci also have been used as indicators of fecal pollution as well as indicating the probable source of contamination. For example, a fecal coliform to fecal streptococci ratio (FC/FS) of 4.4 in a sample would indicate that the water was contaminated with

human fecal material, whereas a FC/FS ratio of 0.1 would indicate contamination from an avian source (American Public Health Association 1985). These indicator organisms have been used in studies to assess wastewater treatment processes (Scheuerman et al. 1987; Legendre et al. 1984; Stetler et al 1984), evaluating underground water quality (El-Zanfaly and Shabaan 1988), and to assess the microbiological quality of bathing and recreational waters (Evison 1988).

Nonbacterial indicators of fecal pollution include bacteriophages of E. coli (Stetler 1984, Havelaar and Pot-Hogeboom 1988) and Bacteroides (Tartera et al. 1989). Bacteriophages are viruses which are specific for their host bacteria, are present in abundance in the human intestine, and cause no pathology to humans. Bacteriophages are excreted in high numbers in fecal material and are found at levels of approximately 200 plaque-forming units (PFU) in 1 ml of raw wastewater (Preston et al. 1989). Coliphages are specific for E. coli and thus are not specific for human fecal contamination of water. Bacteroides is a normal and specific inhabitant of the human intestine, and thus bacteriodies-phage are specific for human fecal contamination of water. Bacteroides is, however, a strict anaerobe and is, therefore, somewhat more difficult to manipulate as a host for bacteriophage assays. Coliphages also have been used to assess the microbiological quality of food (Kennedy et al. 1986; Vaughn and Metcalf 1975) and water (Scheuerman et al. 1987; Bitton et al. 1981). Due to the persistence and the existence of a niche for coliforms and fecal coliforms in Puerto Rican fresh and marine waters (Santiago-Mercado and Hazen 1987; Valdes-Collazo et al. 1987; Perez-Rosas and Hazen 1988; Bermudez and Hazen 1988; Rivera et al. 1988), coliphages have been recommended as indicators of fecal contamination in tropical areas (Hernandez-Delgado and Toranzos 1990). Considering recent events in Central and South America, these findings are of great interest; and further studies should be followed carefully.

In conclusion, it is evident that there are numerous pathogenic microorganisms which may or may not be present in fecally-polluted waters. It would be impossible for field units, or even wastewater treatment facilities, to assay waters for all of these etiological agents. Therefore, indicators of fecal pollution have been used to assess the quality of water. Indicators which would be of value under field conditions include the coliforms, fecal coliforms, fecal streptococci, and bacteriophages of E. coli. More than likely, the use of coliforms and fecal coliforms to assess water quality in tropical regions has led to an excessive number of false-positives. For these regions, coliphages are recommended as the indicator of choice.

Table 1. Bacteria generally found in the healthy human intestine. [From Drasar and Barrow (1985)]

Type of Bacteria	Genera
Anaerobic Gram-negative rods	Bacteroides Desulfomonas

Type of Bacteria	Genera
	Leptotrichia
	Fusobacterium
	Butyrivibrio
	Succinimonas
	Vibrio
Facultative Gram-negative rods	Escherichia
	Citrobacter
	Klebsiella
	Enterobacter
	Proteus
	Lactobacillus
Anaerobic Gram-positive rods	Bifidobacterium
	Clostridium
	Eubacterium
	Lachnospira
	Propionibacterium
Anaerobic Gram-positive cocci	Acidaminococcus
	Megasphaera
	Peptococcus
	Ruminococcus
	Sarcina
	Streptococcus
	Veillonella
	Coprococcus
	Gemmiger

Table 2: Bacteria generally capable of causing acute diarrhea. [From Jawetz et al. (1987)].

Organism	Incubation Period (Hours)	Pathogenesis
Staphylococcus	1-8	Enterotoxin
Bacillus cereus	2-16	Enterotoxin
Clostridium		
perfringins	8-16	Enterotoxin
botulinum	24-96	Exotoxin

Organism	Incubation Period (Hours)	Pathogenesis
dificile	?	Enterotoxin
Escherichia coli (some strains)	24-72	Enterotoxin
Vibrio parahaemolyticus	6-96	Enterotoxin
Shigella	24-72	Epithelial invasion
Salmonella	8-48	GI infection
Campylobacter jejuni	2-10 days	Epithelial invasion
Yersinia enterocolitica	?	Variable

Table 3: Intestinal infections: infective dose in man. [From Drasar and Barrow (1985)].

Organism	Infective Dose
Escherichia coli	10^6-10^8
Salmonella sp.	10^4-10^8
Shigella sp.	10-200
Vibrio cholerae	10^8
Vibrio parahaemolyticus	10^6-10^8

Table 4: Human viruses which may be found in polluted waters. [From Rao and Melnick (1986)].

Virus Group	Number of Serotypes
Poliovirus	3
Echovirus	34
Coxsackievirus A	24
Coxsackievirus B	6

Virus Group	Number of Serotypes
New enteroviruses	4
Hepatitis A	1
Norwalk virus	2
Rotavirus	4
Reovirus	3
Parvovirus	3
Adenoviruses	>30
Cytomegalovirus	1
Papovavirus	1

Table 5: Helminths which can cause human pathology via the consumption of contaminated water or food. [From: Heyneman (1987)].

C=cestode(tapeworm) N=nematode(roundworm) T=trematode(fluke)

Parasite	Location in Host	Geographic Location
Angiostrongylus cantonensis (N)	Meninges	Southwest Pacific
Angiostrongylus costaricensis (N)	Bowel wall	Central America,
Anisakis (N)	Stomach	Pacific basin
Ascaris lumbricoides (N)	Small intestine	Worldwide
Clonorchis sinensis (T)	Liver	China, Korea, Japan, Taiwan, Indochina
Capillaria philippinensis (N)	Small intestine	Philippines, Thailand
Taenia solium (C)	Eye and brain	Worldwide
Dracunculus medinensis (N)	Leg or foot	Africa, Arabia to Pakistan, Asia
Echinococcus granulosus (C)	Liver, lung, brain bones, kidney	Worldwide

C=cestode(tapeworm) N=nematode(roundworm) T=trematode(fluke)

Parasite	Location in Host	Geographic Location
Echinostoma ileocanum (T)	Small intestine	SE Asia
Enterobius vermicularis (N)	Cecum and colon	Worldwide
Fasciola hepatica (T)	Liver	Worldwide
Fasciolopsis buski (T)	Small intestine	E and SE Asia
Gnathostoma spinigerum (N)	Subcutaneous	E and SE Asia, Japan, Egypt
Heterophyes heterophes (T)	Small intestine	China, Korea, Taiwan, Israel
Ancylostoma duodenale (N)	Small intestine	Worldwide tropics and North America
Ancylostoma braziliense (N)	Subcutaneous	Worldwide
Toxocara sp. (N)	Lung, liver, eye, brain, other	Worldwide
Metagonimus yokogawai (T)	Small intestine	As Heterophyes, USSR, Balkans, Spain
Opisthorchis felineus and viverrine (T)	Liver	E. Europe, USSR, Thailand
Paragonimun westermane (T)	Lung, brain, other	E and S Asia, N Africa, S and N America
Schistosoma haematobium (T)	Urinary bladder, large intestine, liver	Africa, Madagascar, Arabia to Lebanon
Schistosoma Japonicum (T)	Small intestine liver	China, Philippines, Japan, Taiwan

C=cestode(tapeworm) N=nematode(roundworm) T=trematode(fluke)

Parasite	Location in Host	Geographic Location
Schistosoma mansoni (T)	Colon, rectum, liver	Africa, S America, Caribbean
Spirometra mansonoides (C)	Wounds	Orient
Strongyloides stercoralis (N)	Duodenum and jejunum	Worldwide
Diphyllobothrium latum (C)	Small intestine	Alaska, E Canada, Great Lakes, Europe, S America, Med., USSR, Japan, Australia
Dipylidium caninum (C)	Small intestine	Worldwide
Hymenolepis diminuta (C)	Small intestine	Worldwide
Hymenolepis nana (C)	Small intestine	Worldwide
Taenia saginata (C)	Small intestine	Worldwide
Taenia solium (C)	Small intestine	Worldwide
Trichinella spiralis (N)	Striated muscle	Worldwide
Trichostrongylus sp. (N)	Small intestine	E Europe, USSR
Trichuris trichiura (N)	Cecum, colon	Worldwide

REVIEW OF CLASSICAL AND NEW DETECTION SYSTEMS

In order to ensure the overall quality of water within the United States, the American Public Health Association, American Water Works Association, and the Water Pollution Control Federation have jointly published the "Standard Methods for the Examination of Water and Wastewater" for which the 17th edition is now available (American Public Health Association 1989). Within this text are included the standard methods, or proposed standard methods, for the examination of water and wastewater for bacteria, viruses, parasites, fungi, and microinvertebrates as determined by the Environmental Protection Agency (EPA). This chapter will review these standard methods and proposed standard methods as well as reviewing current research on these particular methods. These methods also will be evaluated as to their applicability to the rapid microbiological assessment of water quality under field conditions.

Bacteria in water can be pathogenic, be nonpathogenic, or indicate the presence of pathogenic bacteria, viruses, parasites, and the like which can be transmitted by the fecal-oral route. Most existing assays applicable to field use utilize selective and differential media to quantitate bacteria which are indicators of fecal pollution of water. These bacteria are not necessarily pathogenic themselves, but their origins and relative numbers make them a good, although not perfect, indicator of pathogenic bacteria and other etiological agents in water. Due to the constraints of size, storage conditions, and electrical supplies, the Army water quality assessment kit uses membrane filters (MF) to concentrate bacteria from water. Bacteria are entrapped, or immobilized, within the filter matrix via filtration and are capable of subsequent growth. These filters are then placed on a filter pad soaked with a selective and differential growth medium (m-Endo), sealed in a spill-proof container, and incubated at 35^{0} C for 18 to 24 hours. The immobilized bacteria will then grow to form colonies which can be differentiated and quantitated directly on their biochemical characteristics based on the media used to cultivate them. Coliforms will show a characteristic "green sheen" due to the fermentative utilization of lactose. Other quantitative methods which could be adapted for field use are the multiple-tube, most-probable number quantitative assay (MPN) and the one-tube presence-absence (PA) qualitative assay. Direct comparative studies of MF, MPN, and PA detection systems for coliforms indicate that the PA system was the easiest to inoculate and interpret and was more sensitive than the MF and MPN methods (Pipes et al. 1986; Jacobs et al. 1986). The PA and MPN tests, however, require much more incubator space than the MF-based assays. Therefore, MF-based assays are recommended for field use at this time.

Heterotropnic Plate Count Perhaps the simplest method to nonspecifically assess water quality is to determine the heterotrophic plate count of a water sample. This test will quantitate the numbers of bacteria in a water sample capable of growing on a rich medium under aerobic incubation conditions. This test does not indicate the presence of fecal pollution of the water sample, but does give some information as to the total numbers of bacteria present. Due to the extreme lack of information generated by this test, it is not recommended for field use.

Total Coliform Group The coliform group may be detected by their biochemical fingerprint of the fermentative use of the carbohydrate lactose with the concatenate production of gas at 35°C within 24 hours while growing on selective and differential media. Media which are used to assay the coliform group differ from those used for the heterotrophs in that they have a higher ionic strength, contain lactose, have a detergent (to simulate the surfactant characteristics of bile), and contain a pH indicator (to indicate the fermentation of lactose). Coliform colonies are differentiated from noncoliform colonies by a characteristic "green sheen" when cultured on solid media or on a membrane filter.

While the presence of coliforms in water indicates that the water may be contaminated with feces of warm-blooded animals some members of the coliform group have niches other than the warm-blooded gastrointestinal tract; thus this test may give false positives. This is particularly true in tropical regions where coliforms thrive in waters (Santiago-Mercado and Hazen 1987; Valdes-Collazo et al. 1987). Investigators are constantly improving the coliform-MF technique as it is the cornerstone of water and wastewater microbiology. Problems have been identified with this assay including interference of noncoliforms on coliform identification (Burlingame et al. 1984; Franzblau et al. 1984; Chen and Hickey 1986; Doyle et al. 1984). Also, anaerobic incubation of the assay has alleviated problems associated with noncoliform overgrowth and interference (Doyle et al. 1984 and Franzblau et al. 1984). The impact of bacterial swarming may be corrected and sensitivity of assay improved using hydrophobic grid membrane filters (McDaniels et al. 1987, Farber and Sharpe 1984) which also allows the automation of the enumeration procedure (Tsuje and Bussey 1986). The hydrophobic membrane filters also reduce the effect of high concentrations of solid debris in the water sample. Several media also have been evaluated for their ability to recover total coliforms from chemically treated water (Rice et al. 1987); no differences were found between the media tested, except that mT7 media may show significantly lower counts than did the other media tested (Adams et al. 1989). Although the coliform test may give false positives, it is recommended for field use at the present time using the MF-type assay.

Fecal Coliform Group The fecal coliform group may be detected on the same basis as the coliform group, with two important exceptions: (1) temperature: samples are incubated at a much higher temperature, 44°C as compared to the 35°C for the coliforms, which simulates the high internal temperature of the mammalian gut; (2) media: the media used to detect fecal coliforms (EC medium) contains bile salts, which selects for bacteria capable of growing under conditions of the mammalian gut. This test is, therefore, much more selective than the coliform test; but due to the highly selective and differential nature of this medium, some false negatives would be expected. This is especially true considering the recent discovery of "injured" bacteria (Domek et al. 1984; McFeters et al. 1986; Roszak and Colwell 1987; LeChevallier and McFeters 1985; LeChevallier et al. 1985; Walsh and Bissonnette 1989; Gurijala and Alexander 1988) and "viable but nonculturable" bacteria (Roszak and Colwell 1987) in environmental samples. Roth et al. (1988) recently reported that betaine can restore colony formation of osmotically stressed *E. coli*. In addition, it has been determined that fecal coliforms are "grossly inadequate for the detection of recent human fecal

contamination and associated pathogens in both marine and fresh tropical waters" because of the simple fact that E. coli and other fecal coliforms are natural inhabitants of pristine and sea-waters of Puerto Rico (Santiago-Mercado and Hazen 1987; Valdes-Collazo et al. 1987; Perez-Rosas and Hazen 1988; Bermudez and Hazen 1988; Rivera et al. 1988). The tropical conditions of Puerto Rico also enable several other human pathogenic bacteria and fecal indicators such as Streptococcus faecalis (Muniz et al. 1989), Salmonella typhimurium (Jimenez et al. 1989), Klebsiella pneumoniae (Lopez-Torres et al. 1987), and Vibrio cholerae (Perez-Rosas and Hazen 1988) to survive or thrive in these waters. The results of these studies indicate that for different climates, especially those in the tropics and subtropics, different indicators are required to effectively determine fecal contamination of water (Hazen et al. 1987). Despite these shortcomings, the fecal coliform test warrants serious consideration for field use as there is a tentative standard method for a 7-hour fecal coliform test (American Public Health Association 1989). This method is similar to the MF fecal coliform test described above except that different media and a higher incubation temperature (41.5°C) are used. The 7-hour fecal coliform test was recently evaluated (Barnes et al. 1989) and was recommended for use during emergencies or disasters. This type of test is highly recommended for field use and can be readily integrated into the existing test kit. It is theorized that the test may be improved by utilizing enzymatic markers of coliforms and E. coli as described below.

Recently, Access Medical Systems, Inc. (Branford, CT) introduced the Colilert autoanalysis test for total coliforms and E. coli simultaneously and in a single assay (Edberg et al. 1988). The system has been approved by the Environmental Protection Agency (EPA) to assess fecal contamination of tap water after extensive investigation were conducted by several laboratories (Edberg et al. 1988; Lewis and Mak 1989; Covert et al. 1989). The system is metabolically based and uses nonchromogenic substrates of enzymes specific for the coliform group or E. coli. Total coliforms and E. coli metabolize the nonchromogen ONPG (o-nitrophenyl B-D- galactopyranoside) to the yellow colored chromogen ONP (o-nitrophenol) using the enzyme B-galactosidase. The E. coli, a member of the coliform and fecal coliform group, can metabolize the nonchromogen MUG (4-methylumbelliferyl-B-D glucuronide) to the chromogen 4-methylumbelliferone (fluorescent under longwave UV light) using the enzyme B-glucuronidase (Robinson 1984). The system requires 18 to 24 hours of ncubation at 35°C and can be used as a MPN or PA assay system. The only problem with this system in terms of field use would be the high demand for incubator space. This problem may be readily solved using the Colilert liquid media in conjunction with the MF-based assays discussed earlier. This test would use the microbiological test kit now employed by the Army for field evaluation of water quality by simply replacing the m-Endo media with the Colilert media and adding a battery-powered ultraviolet lamp. Coliforms and E. coli could, therefore, be determined with greater precision using existing equipment. A methodology similar to that discussed above was reported by Freier and Hartman (1987) using a system independent of Colilert. The method of Freier and Hartman (1987) also would tend to reduce the problems that are associated with the use of the Colilert system with high turbidity waters as bacteria are allowed to form individual colonies rather that grow in a mixed culture, which has been shown to give false positives with the Colilert system

(Covert et al. 1989). Microcolony analysis such as that used by Rodrigues and Kroll (1989) also may be used to reduce the time required to evaluate water quality using M: techniques. Enzyme-linked assays (EIA) also have been developed to detect E. coli B-galactosidase and B-glucuronidase for the confirmation or identification of environmental isolates (Kaspar et al. 1987). In addition to Access Analytical Systems' Colilert test, Hach Co. (Loveland CO) produces a similar test (coli-MUG) which detects the production of gas from lactose and the cleavage of MUG for the detection of coliforms and E. coli. The system is much more compact than that offered by the Colilert system and may be more applicable to field use. Recently, Berg and Fiksdal (1988) reported the evaluation of fluorogenic substrates for B-galactosidase as well as for B-glucuronidase to detect coliforms and fecal coliforms using an MF based assay. Two new prototype PA media from Milipore Corp. and from the University of Washington for total coliforms and E. coli using chromogenic and fluorogenic indicators will be available in the very near future. These types of systems are highly recommended for use under field conditions and could be readily implemented into the existing field test kit.

Fecal Streptococcus The fecal streptococci may be used as indicators of fecal pollution of water using MPN, PA, and MF techniques. Media which are used for these tests select for salt-tolerant, azide resistant bacteria capable of reducing 2,3,5-triphenyltetrazolium chloride (American Public Health Association 1989). The media used for these tests are highly selective in nature and the resulting colonies tend to be very small and relatively hard to examine after the required incubation of 48 hours at 35°C. The ratio of fecal coliforms to fecal streptococcus (FC/FS) found within a given water sample gives valuable information as to the source of contamination (American Public Health Association 1989). For example, a FC/FS ratio of 4.0 would indicate human contamination whereas a FC/FS ratio of 0.1 would indicate contamination from birds. Intermediate ratios would indicate mixed source contamination. The information generated by the FC/FS ratio has the greatest applicability for the evaluation of field water quality and has a better predictive value of a negative test than coliforms when using the French water standards (Collin et al. 1988). The tests presently available, however, are not suited for field use due to the long incubation times required.

Coliphage and Bacteroides-Phage Another indicator of fecal pollution of water are the coliphage group and Bacteroides phage. Coliphages are viruses which attack the E. coli whereas bacteroides-phage attack Bacteroides, a strict anaerobe whose specific niche is the human gastrointestinal tract (Tartera et al. 1989; Tartera and Jofre 1987). Coliphage detection is a tentative standard method for the examination of water quality. It is relatively simple, and results are obtainable in 4 to 6 hours (American Public Health Association 1989). The numbers of coliphage in a sample can be correlated to the number of coliforms or fecal coliforms by simple logrithmic formulae as shown below (Isbister et al. 1983; American Public Health Association 1989), but different water types and locations should be independently verified (American Public Health Association 1985).

Total coliforms

$$\log(y) = 0.627 \times \log(x) + 1.864$$

where: y = total coliforms/100 ml
x = coliphages/100 ml

Fecal coliforms

$$\log(y) = 0.805 \times \log(x) + 0.895$$

where: y = fecal coliforms/100 ml
x = coliphages/100 ml

The speed of the assay results from the rapid replication of phage within its host cell where one infected cell will be destroyed in 20 minutes with the release of approximately 200 phages. The assay requires viable host cells and melted agar (American Public Health Association 1989). Coliphages also have been used to assess fecal contamination of foods (Kennedy et al. 1986; Kennedy et al. 1984; Kott and Gloyna 1965) and have been proposed as an indicator of enteroviruses in shellfish (Vaughn and Metcalf 1975; Power and Collins 1989) and water (Stetler 1984; Havelaar and Pot-Hogeboom). Phages other than coliphages have been differentiated by DNA/DNA hybridization procedures (Jarvis 1984). Bacteroides-phage has been proposed as an indicator of enteroviruses in water (Tartera et al. 1989). In relatively clean waters, coliphages exist in low numbers, which requires that they first be concentrated (Shields et al. 1986) although methods are available to assay 100 ml of water at a time (Grabow and Coubrough 1986). Several methods have been developed for the concentration of coliphages from water which include magnetite-organic flocculation (Bitton et al. 1981), ammonium sulfate flocculation (Shields and Farrah 1986), and adsorption-recovery of phages to filter materials (Shields et al. 1986; Seeley and Primrose 1979). The use of coliphages to assess water quality, especially viral contamination, is an active area of research and should be watched closely for further developments. This type of assay is highly recommended for field use due to the speed, sensitivity, and very simple nature of the procedure. Problems which may have to be overcome include having a source of host bacteria (lyophilization may or may not be an alternative) and the requirement for anaerobic conditions for the bacteroides-phage assay. Further studies as to the correlation of coliphages to coliforms and fecal coliforms (Hernandez-Delgado and Toranzos 1990), validity of the indicator (Hernandez-Delgado and Toranzos 1990), and water types where this indicator is best suited (Hernandez-Delgado and Toranzos 1990; El-Abagy et al. 1988; Toranzos and Gerba 1988) are expected in the very near future.

Pathogenic Bacteria In addition to the indicator bacteria previously discussed, there are standard methods for the detection of pathogenic bacteria in the environment (American Public Health Association 1989). Methods are available for the detection of Salmonella, Shigella, enteropathogenic E. coli, Campylobacter, Vibrio, Leptospira, Legionellaceae, Yersinia and many others. The literature abounds with techniques and comparisons of media for the detection of these and other human pathogens from food, water, and other

exposure sources to the extent that they will not be reviewed. These techniques require specific media for each microorganism and are not recommended for the examination of field water quality using presently available technology.

Pathogenic Protozoa Pathogenic protozoa such as Giardia lamblia (Rose et al. 1989; Kong et al. 1988; DeRegnier et al. 1989) and Cryptosporidium (Fayer and Ungar 1986; Peeters et al. 1989) persist as environmentally resistant cysts at low levels in waters polluted by humans or wildlife (Porter et al. 1988; Ongerth and Stibbs 1987; Pacha et al. 1987; Erlandsen et al. 1988), but have a low infective dose of approximately 10 cysts (Rendtorff 1954). Due to low levels of these cysts in water and their low infective dose, these cysts must first be concentrated prior to detection. Following standard methods (American Public Health Association 1989), cysts are collected on a filter cartridge made of wound yarn with a 1 um pore size. Other investigators have used polypropylene filters (Musial et al. 1987) or tangential-flow filtration (Isaac-Renton et al. 1986) to concentrate cysts from water. Cysts which are entrapped within the filter material must then be removed by manually taking apart the filter and kneading the material in 1 liter of distilled water or a Tween solution (American Public Health Association 1989). Cysts are further concentrated by zinc sulfate density centrifugation at 650 x g for 3 minutes. The cysts will band at the meniscus, are collected on a microscope slide, and are then determined by "an individual with a demonstrated proficiency for protozoa" (American Public Health Association 1985). The cysts can be examined by direct microscopy (American Public Health Association 1989), phase-contrast microscopy (Sauch 1985), or immunofluorescent microscopy (Sauch 1985; Rose et al. 1989). Using present technology, these techniques are obviously not recommended for field use.

From previous discussions it is quite evident that there are several types of parasites that can be present in waters besides Giardia and Cryptosporidium. There are, however, no standard methods listed for the detection of these microorganisms besides microscopic examination charts (American Public Health Association 1989). Recently, due to the proliferation of nuclear power plants and an interest in the public health and ecological effects of artificial warming of waters by power-plant effluents, studies have focused on the ecology of thermophilic amoebae and pathogenic Naegleria fowleri in water (Tyndall et al. 1989). High numbers of pathogenic amoebae were found in warmer waters and sediments, and persons residing in warmer climates have higher antibody titers against these microorganisms (Tyndall et al. 1989). Free-living pathogenic and nonpathogenic amoebae also have been investigated and found at elevated levels in soils and waters receiving municipal wastewater effluents and sludges (Sawyer 1989). In order to detect these microorganisms, the water sample must first be concentrated on a course filter and then cultivated on a lawn of E. coli for 7 days. Amoebae are then identified by microscopy. As with Giardia and Cryptosporidium, a highly trained individual is required to properly identify these microorganisms. These procedures are not recommended for field evaluation of water using presently available technologies.

Enteroviruses Like the pathogenic protozoan cysts previously discussed, enteroviruses persist for extended periods of time within the environment and

have a low infective dose of approximately 1 to 100 infectious particles (Berg et al. 1976). This situation is complicated by the fact that individuals may have apparent or inapparent infections, and that infected individuals can shed up to 10^6 infectious virus particles per gram of fecal material (Rao 1982), which leads to an explosive epidemiological cycle which is difficult to control. The first step in detecting enteroviruses is the concentration of viruses from water. Sample size should be at least 40 liters of potable water when considering the average per-capita exposure to water in the United States (American Public Health Association 1989). A simple field method for concentrating viruses from water has been developed (Toranzos et al. 1984) Several tentative standard methods exist for the concentration of viruses from water (large and small volumes) and solids (American Public Health Association 1989, Block and Schwartzbrod 1989). These methods include: 1) microporous filter adsorption-elution methods for concentrating viruses from small volumes of water using electronegative filters such as cellulose nitrate or fiberglass, 2) microporous filter adsorption-elution method for concentrating viruses form large volumes of water and treated wastewater using electronegative filters, 3) virus concentration by aluminum hydroxide adsorption-precipitation for small volumes of water, 4) hydroextraction-dialysis with polyethylene glycol for very small volumes of water, and 5) recovery of viruses from suspended solids in water and wastewater by eluting adsorbed viruses from the suspended solids. One novel method which has been evaluated for the recovery of fish viruses is molecular filtration (Watanabe et al. 1988). The literature abounds with methods to concentrate viruses from other sources such as raw shell fish (Speirs et al. 1987; Lewis and Metcalf 1988), sewage (Payment et al. 1979; Hejkal et al. 1984; Poyry et al. 1988), sludges (Rao and Melnick 1986; Goyal et al. 1984; Berg et al. 1982; Berg and Sullivan 1988; Safferman et al. 1988), fecal material (Pontefract and Bergeron 1985), sediments (Goyal et al. 1984; Rao et al. 1984; Bitton et al. 1982; Lewis and Metcalf 1988), seawater (Goyal et al. 1984; Lewis and Metcalf 1988), and tapwater/drinking water (Payment and Trudel 1984; Melnick et al. 1984; Keswick et al 1984; Smith and Gerba 1982; Sobsey et al. 1985; Lewis and Metcalf 1988; Toranzos and Gerba 1989). A central theme of methods to concentrate viruses from large volumes of water is that viruses are adsorbed to a solid matrix under specific conditions of pH, ionic strength, and the type and concentration of salts in solution. When dealing with 40 liters or more of water, these adjustment can be somewhat troublesome. Electronegative filters require extensive conditioning of the water, whereas electropositive filters generally do not (Rose et al. 1984; Shields et al. 1985; Sobsey and Jones 1979; Sobsey and Glass 1980; Preston et al. 1989; Preston et al. 1988). Once viruses are adsorbed onto the filter material, they are eluted using a complex proteinaceous solution such as beef extract (Block and Schwartzbrod 1989; Rao and Melnick 1986). The resulting eluent is then further concentrated to a more manageable volume using some sort of secondary concentration procedure which usually relies upon the precipitation of the eluent along with any viruses within the sample. This precipitate is then suspended into physiological buffer for tissue culture assay using tentative standard methods (American Public Health Association 1989). At this point, the sample may be contaminated with bacteria, fungi, or indigenous toxins which must be dealt with prior to the use of tissue culture (American Public Health Association 1989). Based on the requirement for extensive sample concentration and tissue culture, the routine assay of viruses in field

water using present technologies is not recommended. Other aspects of viruses detection in water make these assays even less applicable to field use and are reviewed below.

The assay and identification of viruses in a sample is an expensive, time consuming, troublesome procedure which requires highly trained individuals. The assay can take up to two weeks to obtain results and at the present time requires the use of tissue culture, which would be all but impossible to maintain under field conditions. A recent study (Preston 1989) compared novel methods for the detection of viruses in environmental samples which would not require the use of tissue culture. Methods which were evaluated included enzyme-linked immunoassays (EIA) using monoclonal, polyclonal, and pooled human serum as the primary antibody, and nucleic-acid hybridization procedures. The results of these studies were somewhat disappointing in that it was revealed that sensitivity is low. Approximately 10^4 to 10^5 plaque-forming units (PFU) of poliovirus would be required for a positive signal using both of these procedures, which is supported by other studies (Deng and Cliver 1984; Rotbart 1989). Nasser and Metcalf (1987) recently reported an amplified enzyme-linked immunosorbent assay (ELISA) for the detection of hepatitis A virus which was five times more sensitive than traditional immuno-assays. For the immunological studies, it was noted that monoclonal antibodies showed much less affinity for a given sample than did polyclonal serum, a well-documented drawback of monoclonal antibody technology (Zam 1989). For epidemiological reasons, pooled human serum showed the greatest potential for immunological techniques. Assuming that serum pooled from a given geographical location or population would have antibody titers against etiological agents in that area, this would be a much better source of antibody than would be rabbit-raised monospecific polyclonal serum or monoclonal antibody preparations (Preston 1989). Tissue culture was found to be an effective method to enhance the numbers of viral antigens present in a given sample, and in situ immunoperoxidase methods have been developed to stain cells infected with viruses from environmental samples (Preston 1989; Payment and Trudel 1985). As with other enzyme-linked assays, pooled human serum was shown to be most suited for these types of assays. Although these EIA procedures can be performed in approximately three to four hours (Deng and Cliver 1984), the sensitivity of the assay requires the use of tissue culture to enhance viral antigens (Preston 1989; Payment and Trudel 1985), and is therefore not recommended for field use at this time using present technology. It is doubtful that these limitations will be overcome in the near future.

As stated previously, nucleic acid hybridization procedures using present technology have a sensitivity of approximately 10^4 to 10^5 PFU of poliovirus and are well within the expected sensitivity of radiolabled dot blot hybridization procedures (Gurley 1989; Kew 1989). These procedures, however, have received significant attention in the clinical arena and were the topic of an extensive review (Rotbart 1989). These procedures are more suited towards clinical than environmental applications due to the higher numbers of virus particles in clinical samples. Several investigators have reported the use of nucleic-acid probes to detect poliovirus (Preston et al. 1989), rotavirus (DeLeon 1989), and hepatitis type A virus (Jiang et al. 1986; Jiang et al. 1987) in environmental samples, and this has been proposed as a possible standard method for the detection of viruses in the environment

(Margolin et al. 1985). There is some dispute as to methods to extract nucleic acids from environmental samples due to high background noise generated by environmental contaminants not normally found when using techniques which were developed for use with tissues or pure cultures. Methods which have been used include protiase K (Ricardson et al. 1988; Margolin et al. 1985), formaldehyde (Preston et al. 1989), beta-mercaptoethanol (Preston et al. 1989), and formaldehyde with beta-mercaptoethanol (Preston et al. 1989). Most viruses which are transmitted by the fecal-oral route contain RNA, which is very susceptible to enzymatic digestion by RNA'ases. RNA'ases exist in high concentrations on the human skin, and one fingerprint can degrade the RNA within a sample in a matter of a few seconds (Sambrook et al. 1989). RNA'ases are immune to autoclaving, but may be inactivated by baking at 180^{0}C for 8 hours or so (Sambrook et al. 1989). Biological inhibitors of RNA'ases are available such as vanainyl ribonuclease inhibitor or human placental RNA'ase inhibitor. Chemical inhibitors include formaldehyde and beta-mercaptoethanol (Sambrook et al. 1989) and have been used for environmental samples (Preston et al. 1989). Virus infection of tissue culture has been found to be an effective method to increase the number of copies of viral nucleic acids within a sample (Preston 1989; Rotbart 1989; Rico-Hesse et al. 1987). Infected cells can be detected by in situ hybridization (Rotbart 1989) or by dot blot hybridization after extracting viral nucleic (Preston 1989; Rotbart 1989; Rico-Hesse 1987). Using present technology, these procedures are probably best suited for the exceptionally specific identification of viruses from the environment (Preston 1989; Preston et al. 1989; Rico-Hesse et al. 1987). Recently, the Cetus Corporation (Emitsville, CA) released an enzymatic method of specifically and logarythmically amplifying the amount of DNA within a sample. This method is known as polymerase chain-reaction (PCR) and it is receiving significant attention for the detection of rare or low-copy genes (Sambrook et al. 1989) and to trace genetically engineered microorganisms (GEMS) in environmental samples (Chaudhury et al. 1989; Steffan and Atlas 1988). This type of method should prove to be applicable to field use for the detection of bacteria, viruses, parasites, and fungi in environmental samples, provided that testing personnel know what to look for. The manufacturer indicates that the system is very simple to use however, a recent advertisement in Science by Academic Press for PCR Protocols promises the book will help "avoid PCR hell." Due to the problems associated with the detection of viruses in environmental samples, it is recommended that coliphages be considered as a rapid assay indicator of viruses in environmental samples. Coliphages were discussed and evaluated earlier in this text.

Pathogenic Fungi Fungi are ubiquitously distributed microorganisms which may be found wherever nonliving organic matter occurs, although some species are pathogenic or parasitic to humans (American Public Health Association 1985). Routine isolations of fungi from polluted streams and wastewater treatment plants have yielded very few pathogenic species of fungi and are therefore of little public health concern. One fungus which is a problem in the tropics is Exophiala mansonii. Other types of fungi which normally reside in the soil can be isolated from streams, probably due to run-off (American Public Health Association 1985). Yeasts, such as Candida albicans, are commensal to humans and pose little to no public health threat. Due to the very low threat that oral or fecal-oral transmitted fungi pose, and the plethora of indicators

available to determine water quality, the detection of fungi in water in the field is not recommended.

Microinvertebrates Microinvertebrates are important to the ecology of bodies of surface water as well as to determining general water quality. They are nonpathogenic but do cause taste, odor, and coloration problems. Pathogenic microorganisms ingested by microinvertebrates may pose a minimal public health threat as they may evade disinfection procedures (King et al. 1988). Applying the logic used for fungi above, microinvertebrates have minimal importance for the evaluation of field water quality.

In conclusion, the following recommendations are made to enhance the capabilities of the existing field water microbiological testing kit. For temperate climates, MF based assays using a biochemical fluorescent marker such as the Colilert test media (Edberg et al. 1988), the system described by Berg and Fiksdal (1988), or a new procedure being developed by the U.S. Environmental Protection Agency, Environmental Monitoring and Support laboratory are recommended. This type of system is readily integrated into the existing field kit and promises to detect 1 coliform in 100ml of sample in 18 hours. The only modifications required are: 1) changing the media, and 2) adding a UV light source to the kit. This type of system can also be modified to detect other groups of bacteria based on their enzymatic signatures by using different fluorescent markers. For tropical climates, a coliphage assay is recommended based on the ecology of coliforms and fecal coliforms in fresh and marine tropical waters. Fluorescent biochemical markers may be included into the coliphage assay to enhance the visualization of plaques in much the same way as these markers enhance the visualization of bacterial colonies. Some evaluation is required for each of these tests to ensure that they meet the requirements of the O&O plan.

NOVEL NEW APPROACHES FOR MICROBIAL DETECTION

Within the past decade, there has been a virtual explosion in molecular, biochemical, and immunological methods for the detection of microorganisms of all types from a wide variety of ecosystems. At the present time, most of these methods as published are not adequate in terms of speed, sensitivity, educational and training requirements, or hardware to be suited for field use. These limitations are presently being addressed in the current literature as well as by several corporations. This chapter will review these novel techniques as they apply to the determination of water quality under field conditions, with an open eye to those techniques which may be adaptable to field conditions in the near future.

Total Bacterial Numbers Recently, several techniques have been introduced which can detect the total bacterial numbers via biochemical staining techniques. Certain dyes, such as acridine orange (Nishino 1986; Bergstrom et al. 1986; Rozak and Colwell 1987; Kogure et al 1987), 4',6'-diamidino-2-phenylindole (King and Parker 1988; Schallenberg et al. 1989), and acriflavin (Bergstrom et al 1986) fluoresce in the presence of DNA or RNA. This phenomenon has been used to directly count viable bacteria in water samples using fluorescent microscopy. This test would be useful under field conditions if only the total numbers of bacteria are of interest, and

the requirement of a fluorescent microscope may be prohibitive. Therefore, these tests are not recommended at this time, but may warrant further investigation in the future. One avenue of future investigation could be the staining of MF assays with these dyes after a short incubation period to determine the total number of colonies produced on that particular medium.

Total Metabolic Activity Total metabolic activity has been investigated to assess bacteriological numbers by such methods as thymidine incorporation (Jeffrey and Paul 1986; Jeffrey and Paul 1988; Smits and Riemann 1988), radioactive carbon uptake (Iriberri et al. 1987; Reasoner and Geldreich 1989), simultaneous incorporation of thymidine and leucine (Chin-Leo and Kirchman 1988), p-iodonitrotetrazolium (INT) reduction to a colored product (Jeffrey and Paul 1986), 2-(p-iodophenyl)-3-p-(nitrophenyl)-5 phenyltetrazolium (INP) dehydrogenation to a colored product (King and Parker 1988), ATP bioluminescence (Tuncan and Martin 1987; Busey and Tsuji 1986), and ATP/DNA ratios (Jeffrey and Paul 1986). There is also a tentative standard method which utilizes bioluminescence to determine total viable microbial load (American Public Health Association 1989). Again, these techniques are applicable to field use only if total numbers of bacteria are of interest, although specificity may be achieved using techniques based on specific carbon source utilization (Reasoner and Geldreich 1989). The INT and INP tests, however, may have some applicability for a very rapid assay of water quality under conditions where it would be desirable for an individual to assay water quality. INT is reduced from a colorless compound to a red precipitate by metabolic reducing forces present in all cells. If microorganisms are immobilized on a filter, exposed to INT (or a similar compound which will bind to the microorganisms but not the filter) and incubated for a short time, precipitated INT will be present on the filter. If high enough levels of microorganisms are present on the filter, it would take on a pink or red hue. Alternatively, the precipitated INT could be recovered with a solvent and read spectrophotometrically. Similar systems could potentially be developed for the DNA/RNA or cell surface binding dyes. These tests warrant further investigation for the possible development of an individual overall water quality test kit, but at the present time are not recommended for field use due to a lack of sensitivity.

Specific Enzyme Activity Measuring the specific enzyme activity of an environmental sample has also been used to assess the bacteriological quality of environmental or food samples. Enzymes which have been investigated include B-glucosidase (King 1986), B-galactosidase (Berg and Fiksdal 1988; Gilliland and Lara 1988), B-glucuronidase (Watkins et al 1988), lipases (Kouker and Jaeger 1987), amino peptidases (Castro et al 1988), nitrate reduction (Hordijk et al 1987; Abeliovich 1987), ferric ion reduction (Lovley and Phillips 1987), multilocus enzyme electrophoresis (Selander et al. 1986), and coagulase (Klapes and Vesley 1986). These tests do warrant further investigation, but are not recommended for field use at the present time. However, when enzymatic tests utilizing fluorogenic substrates are linked to bacterial colony formation using MF technology, a rapid and sensitive assay suitable for field use can result (Berg and Fiksdal 1988).

Particle Counters Particle counting and other physical methods to determine bacterial numbers or activity have gained increasing attention in recent

years. Flow cytometry has been used to detect bacteria (Donnelly and Baigent 1986; Yagoda-Shagam et al. 1988), potentiometric assays to detect lipoic acid reduction of indicator organisms (Charriere et al. 1984), particle counters to determine bacterial biomass in seawater (Kogure and Koike 1987), and immersible probes for the continual monitoring of microorganism population density (Maxwell et al. 1987). At the present time, these techniques lack the sensitivity requirements for field use. They do, however, show promise for future investigation if the numbers of bacteria can be increased by a simple incubation step. These techniques have the notabled advantages of being very simple to operate as well as giving an output which is very visual and rapidly interpreted. Techniques which allow the determination of a specific group of bacteria based on their surface antigens (Donnely and Baigent 1986) or specific metabolism (Charriere et al. 1984) show the greatest promise. Techniques which show an increase in bacterial mass in selective media (Kogure and Kioke 1987; Maxwell et al. 1987) also show promise for future investigation.

Molecular Methods Recently, several investigators have been developing molecular methods for the detection of pathogenic microorganisms in food and water. Molecular techniques include plasmid analysis (Lobb and Rhoades 1987; Dodd et al. 1988; Whiley et al. 1988), plasmid fingerprinting (Kakoyiannis et al. 1984), DNA/DNA hybridization (Morris et al. 1987; Kuritza et al. 1986; Notermans et al. 1989; Barkay et al. 1989), DNA colony hybridization (Jagow and Hill 1986; Datta et al. 1987; Zeph and Stotzky 1989; Morotomi et al. 1988), DNA/RNA hybridization (Nakhforoosh and Rose 1989; Preston et al. 1989), RNA/RNA hybridization (Shieh et al. 1989), and ribosomal RNA (rRNA)/DNA hybridization (Festl et al. 1986) for the detection of bacteria, viruses, and parasites in food and water samples. The use of gene probes for the study of viruses in the environment was reviewed in Chapter 2.

Despite the enormous amount of press that molecular techniques have attained due to their specificity and as controversial laboratory tools, several aspects of these techniques do not make them amenable to field use with existing technology. Some of these limitations will be reviewed here. An important aspect of the applicability of molecular techniques to environmental samples is the rapid, efficient, and safe recovery of nucleic acids, in some cases both DNA and RNA, from water and soil samples. Several techniques are available (Somerville et al. 1989; Steffan et al. 1988; Preston et al. 1989), most of which require extensive training and hazardous chemicals. It is anticipated that extraction methods which could be used under field conditions will be available within the next few years. Other major problems associated with molecular techniques are the sensitivity (approximately 10,000 microorganisms using radiolabled probes) and the speed (approximately 70 hours) of the assay.

In terms of the senstivity of molecular techniques, ribosomal RNA (rRNA) hybridization based assays and PCR amplification techniques are the most encouraging. The studies of Festl et al. (1986) should be seriously considered as rRNA exists at levels of approximately 10,000 copies per healthy cell and is at least genus specific based on bacterial evolutionary studies (Woese 1987). Ribosomal RNA (rRNA), unlike messanger RNA (mRNA) is a very stable molecule due to its extensive secondary structure. The polymerase

chain reaction (PCR) methodologies discussed in Chapter 2 can specifically enhance the number of copies of a DNA sequence 1 million-fold overnight from virtually any sample (Sambrook et al. 1989). As such, PCR may appear to solve most, if not all of the problems associated with molecular techniques. PCR technology has received a great deal of press, including publishers promising that their book will help "avoid PCR hell", which tends to dispute the manufacturer's claims of the simplicity of the technique. One drawback of PCR technology is that it is presently set for only DNA samples. RNA, a very difficult molecule to work with, may be converted into DNA samples by reverse transcriptase, a rather finicky enzyme under the best of conditions. Both of these systems warrant serious consideration despite their limitations.

Great strides are also being made in detecting positive hybridization reactions using non-radioactive systems as well as increasing the speed of the assay. Novel systems are relying on liquid-phase rather than traditional solid-phase systems. The liquid-phase systems allow a more kinetically favorable condition for hybridization and is therefore much more rapid and sensitive that the solid-phase system. Non-radioactive detection systems based on intercalating dyes and enzymatically-labeled probes (Zeph and Stotzky 1989) are also being developed which greatly simplify the procedures.

In conclusion, molecular techniques for the detection of pathogenic or indicator microorganisms in the environment are at present very time consuming, require radioactive labels for sensitivity, require high levels of education and skill, and therefore, would be difficult to implement under field conditions. Basic research on the industrial level, especially systems involving PCR and rRNA hybridization, will soon make these procedures amenable to field use. Several corporations are already claiming the development of systems which are capable of detecting 100 microorganisms within 2 hours from clinical samples. These systems should be available between 1990 and 1992 and their evaluation should be of very high priority.

Immunological Techniques Investigators are also focusing on immunological techniques to detect pathogenic microorganisms. Methods which have been receiving significant attention include the immunoperoxidase staining of flagella (Hall and Krieg 1984), immunofluorescent microscopy (Hugenholtz et al. 1987; Germida 1984; Koch et al. 1986), filter paper immuno-dot assays (Fletcher 1987; Wright and Morton 1989), membrane filter immunoassay (Cerqueira-Campos et al. 1986; Berube et al. 1989), hydrophobic membrane grid immuno-assay for E. coli (Todd et al. 1989), immuno-toxin detection (Thompson et al. 1986; Jackson et al. 1986; Edwin et al. 1986), enzyme immuno-assays (EIA) (Ayanaba et al. 1986; Riche et al. 1988), enzyme capture assays for E. coli (Holt et al. 1989), and fluorescent antibody direct viable count (Brayton et al. 1987). In addition, an immunofluorescence-DNA-fluorescence staining technique has been developed (Muyzer et al. 1987). Sources of antibodies may be monoclonal sera, polycolonal sera raised in laboratory mammals, pooled human serum for epidemiological studies, or for a novel change of pace, chicken egg yolk from immunized chicken hens (Ricke et al. 1988). The use of immunized chicken egg yolk as a source of antibodies is a very uninvasive and efficient method of obtaining polyclonal antibody preparations and should be highly considered when contemplating immunological studies. In general, immunological methods are not well suited for field use. Problems associated

with these assays include: 1) antibodies have relatively short shelf life and require refrigeration, 2) the sensitivity is approximately 10,000 microorganisms, and 3) non-fluorescent detection systems require the use of carcinogenic and unstable detection reagents. Using present technologies, these procedures are not recommended for field use. These techniques are very powerful in a research or clinical setting, but lack the ability to integrate readily into field applications at the present time, especially considering the poor stability of antibody preparations and the storage conditions presented in the O&O plan. Should the kit be stored at a constant -20°C, these techniques could be made more feasable. One method that may be considered is use of fluorescent labeled primary or secondary antibodies against bacterial species of interest to stain MF based assays after a short period of incubation to allow enhanced visualization of colonies or microcolonies under a hand-held UV light source.

CONCLUSIONS AND RECOMMENDATIONS

This chapter will concentrate on recommendations for short- and long-term modifications to the existing field water testing kit being employed by the Army. Commercially available techniques which fit or can be modified to fit the O&O plan will be evaluated on several criteria: 1) speed of the assay, 2) sensitivity of the assay, 3) ease of operation, 4) educational requirements, and 5) ability of the assay to fit into the existing O&O plan. The intent of these evaluations is to: 1) provide a system which could be integrated into the existing field water test kit with very few modifications, and 2) to evaluate up-and-coming technologies which may integrate into the existing test kit or replace the kit entirely.

Upon reviewing the literature, it became evident that three broad methodologies could be used to reduce the time requirements for field water analysis. These methodologies are: 1) biochemical, 2) immunological, and 3) nucleic acid hybridization. Biochemical tests would detect enzymes specific for a single bacterium or group of bacteria with chromogenic or fluorogenic indicators and would, therefore, not require extensive incubation, especially when considering fluorogenic indicators. Immunological techniques would rely on the specificity of the antigen-antibody recognition event and enzymatic or fluorogenic markers to visualize microorganisms. Nucleic acid hybridization techniques would rely on the specificity of DNA or RNA sequences for a specific microorganism or group of microorganisms for detection purposes. Of these three techniques, biochemical methodologies are recommended for short-term modifications of the field water test kit based on this literature review, personal experience, and a market survey of available techniques. For long-term modifications of the field water test kit, nucleic acid hybridization methodologies are recommended for future use based on this literature review, personal experience, and a market survey of available techniques as well as those techniques available in the very near future.

The presently employed field water microbiological test kit utilizes membrane filters (MF) to immobilize bacteria from 100 ml water samples. This MF is then placed on m-Endo media in an appropriately sized, tight sealing

petri dish and incubated at 35°C for 18 hours. After this incubation period, the MF is observed for colonies showing a green sheen, which is indicative of coliform bacteria due to the fermentative utilization of lactose.

Short Term Improvements for Water Monitoring Recommendations for short-term modifications to the field water microbiological test kit are as follows. Access Medical Systems, Inc. (Branford, CT) recently introduced the Colilert test for total coliforms as well as Escherichia coli in water (Edberg et al. 1988). Similar systems, Coli-Quik (Hach Corp.), Coli-Sure (Millipore Corp.), and Coli-MUG (Hach Corp.), have since then been introduced but these have not been as extensively documented for their efficacy as the Colilert system. These systems are exceptionally simple to use, a water sample is placed into replicates of 10 ml tubes for a most-probable number (MPN) assay or into one 100 ml bottle for a presence/absence (PA) assay and incubated for 18 to 24 hours at 35°C. After this time, bacteria present in the sample, as well as their enzymes, will increase logarythmically with time. Within the media are two compounds which are colorless and do not fluoresce under UV light. When B-galactosidase, an enzyme specific for the coliform group which is responsible for the utilization of lactose, is present in the media, one of the compounds is cleaved to produce a yellow chromogen. When B-glucuronidase, an enzyme specific for E. coli, is present in the media, the other compound is cleaved to produce a fluorescent chromogen. Therefore, tubes are positive for coliforms if the media turn yellow, and positive for E. coli if it fluoresces under UV light. This test is expected to be approved for the examination of drinking waters for coliforms and E. coli by the Environmental Protection Agency (EPA) after extensive evaluation by several research groups (Edberg et al. 1988; Lewis and Mak 1989; Covert et al. 1989). This development has stimulated research into biochemical methods for the detection of bacteria in water. Recently, Berg and Fiksdal (1988) reported the development of fluorescent chromogens for the detection of B-galactosidase, and therefore coliforms, using a MF type assay. This assay can detect one coliform in 100 ml of water after 6 hours of incubation at 35°C. The only requirements for the direct integration of this test into the existing field water microbiological test kit is changing the media to that used by Colilert (Edberg et al. 1988) and modified by Berg and Fiksdal (1988) and the inclusion of a long-wave UV light source. There is one additional safety feature that must be included: safety glasses must be worn by the user to prevent UV mediated damage to the retina. One minor problem that may have to be considered is the inclusion of Berg and Fiksdal's (1988) fluorescent chromogen into the Colilert media. A simple solution would be to have the field operator simply add the additional chromogen to the Colilert media. Both the Colilert and Coli-MUG systems should be investigated for adaptablility to the method of Berg and Fiksdal (1988) in terms of the speed, sensitivity, and types of water which can be tested with confidence as well as the willingness of Access Medical, Inc., Millipore Corp. and Hach, Co. to modify their media to suit the Army's needs.

When considering tropical regions, coliforms and fecal coliform have been shown to be poor indicators of fecal pollution of waters (Hazen et al. 1987). Coliphages have been investigated as an alternative indicator (Hernandez-Delgado and Toranzos 1990). From personal experience, it is known that the coliphage assay is very simple to perform and that results are

obtainable in 4 to 6 hours. The only problems associated with this type of assay under field conditions is the requirement that a viable host strain of E. coli be provided and an agar-based media be prepared. Lyophylized preparations of E. coli may allow extended storage of the host strain and a very small microwave oven or thermal coil could be used to remelt agar which has been previously prepared by autoclaving. A microwave oven is recommended over large volumes of boiling water to melt agar for safety considerations. Another alternative is to use solidifying agents that do not require high temperatures and that can be catalysed to harden by chemical means (eg. addition of cation). Some research may be provided to enhance the visualization of plaques for this assay. The fluorogenic markers for E. coli discussed above and the dyes specific for metabolically active bacteria would be prime candidates for this task. Another area of improvement for the coliphage assay system is the incorporation of a pH 6.0 buffer to samples prior to filtration for optimum retention on the filters.

Long Term Improvements for Water Monitoring Recommendations for long-term modifications to the field water microbiological test kit using novel techniques and technologies are as follows. Of the methods examined in this review, those utilizing nucleic acid hybridization reactions promise to reduce the time requirements for assaying the microbiological quality of water to less than five hours. The specificity of nucleic-acid hybridization reactions makes them very attractive for the identification of a specific species or group of related species and can therefore be used to detect bacteria, viruses, parasites, and the like. The major problems associated with these techniques using presently available technologies are: 1) the sensitivity of the assay (approximately 10^4 microorganisms are currently required), and 2) the speed of the assay (currently 12 to 72 hours are typically needed). These techniques will require extensive modification and evaluation before they can be used under field conditions.

Two up-and-coming methodologies would allow a selective amplification of the desired nucleic acid sequences. These techniques are: 1) the utilization of rRNA as the target sequence (Festl et al. 1986), and 2) selective amplification of target sequences using PCR technology (Sambrook et al. 1989). Since rRNA is specific for a given species or group of species depending on their relatedness, these assays should be specific for the desired organism or group of organisms. An added advantage of rRNA as a target sequence is that there are approximately 10^4 copies of rRNA per cell. This type of approach would reduce the need for the number of organisms within a water sample to be enhanced by techniques as selected enrichment. These techniques would be suitable for the detection of bacteria, parasites, and other eukaryotic organisms, but unsuitable for the detection of waterborne viruses as they do not possess rRNA. Some viruses, such as the pox viruses, do possess rRNA due to "sloppy" assembly within the host cell. PCR techniques allow a 10^6-fold increase of a desired nucleic-acid sequence utilizing a thermostable DNA dependent DNA polymerase and specific primers. Samples which contain a desired nucleic acid sequence at very low levels can therefore be selectively amplified to a level detectable by hybridization procedures. It is very possible that this technique would not be applicable to the detection of RNA-containing viruses such as polioviruses and hepatitis type A virus as an additional enzymatic step would be required to convert RNA to DNA for the

amplification procedure to proceed. The enzyme required is reverse transcriptase and is difficult to work with even under exacting laboratory conditions. One other problem associated with RNA (other than rRNA) is that it is susceptible to RNA'ses. RNA'ses are found at high levels in fingerprints and other anthropogenic sources, which makes operator error very high even among experienced users. rRNA is actually resistant to RNA'se activity due to its extensive secondary structure.

There has been a recent trend in molecular biology to reduce the time required to detect a positive hybridization reaction as well as moving away from radiolabeled probes as a detection system. Presently, radiolabeled probes are required for optimal sensitivity. Hybridization reactions can take place in a solid- or liquid-phase. Liquid-phase hybridizations are gaining popularity as they are much more kinetically favorable than solid-phase systems and are therefore much more sensitive and rapid. Enzymatic and immunological methods have been introduced to reduce the requirement for radiolabled probes for the detection of hybridization events. These techniques, however, are approximately 10-times less sensitive than radiolabeled systems and are not recommended for field use at this stage of the technology. One very interesting and simple detection system is the use of dyes which intercalate into double-stranded DNA, are therefore removed from the bulk solution, and fluoresce under UV light. Dye which is not bound by the double-stranded DNA is then chemically hydrolyzed to a non-fluorescent end-product. Positive hybridization is therefore determined by fluorescence under UV light. This type of system is highly recommended due to its simplicity and speed of determination.

Immunological techniques are not recommended for field use at this time due to the instability of immunoglobulins. Antibody preparations must be stored at -20^{o}C if their activity is to be maintained for even short periods of time. Immunological techniques are, however, very specific, rapid, and easy to use and understand. If the storage requirements can be made less stringent in the future, these techniques may have a place in the field evaluation of water quality.

In brief review, biochemical techniques utilizing fluorescent metabolic markers, short incubation periods, and MF-based assays are recommended for short-term modifications to the existing field water test kit in temperate climates. These modifications are very "non-invasive" to the existing test kit, the only additional equipment necessary is a UV light source, and some results are obtainable in 6 hours. For tropical climates, assays for coliphages in water are recommended for short-term modifications to the test kit. This system is likewise "non-invasive" to the existing test kit. Modifications require a source of host bacteria (*E. coli*) and the capability to melt agar (such as a small microwave oven, steam bath, or boiling water). Any method which would fall under the category of short-term modifications should center upon organisms which are indicators of fecal pollution of water (such as the coliforms, fecal coliforms, fecal streptococci, coliphages, or *Bacteroides* phages) rather than etiological agents themselves. However, systems using fluorescent metabolic markers could be tailored to any desired bacterium or group of bacteria, depending on the availability of the desired fluorescent metabolic substrate.

Nucleic acid hybridization procedures using rRNA as the target sequence, or PCR to selectively amplify a target sequence, are recommended for long-term modifications to the field water test kit. The hybridization detection procedure which shows the greatest promise is the use of fluorescent intercalating dyes. These long-term modifications to the field water testing kit could be used to detect specific etiological agents as long as a "bank" of probes against agents of interest is initiated and maintained.

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